HaCaT Cell Line as a Model System for Vitamin D_3 Metabolism in Human Skin

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Synthesis and catabolism of calcitriol ($1,25(OH)_2D_3$) were studied using HaCaT cell line as a cell culture model. Our results indicate that stimulation of HaCaT cells with epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) within 16 h just prior to reaching confluence amplified the production of calcitriol when calcidiol (^{3}H-25OHD$_3$) was used as a substrate. EGF- and TGF-α–induced (0.1–10 nM) 1-hydroxylation of ^{3}H-25OHD$_3$ was concentration-dependent but showed different kinetics. Synthesis of calcitriol induced by EGF was inversely related to the degree of cellular confluence. Stimulation by EGF was an actinomycin D- and cycloheximide-sensitive process. Independently of the growth factor used, the production of ^{3}H-24R,25(OH)$_2$D$_3$ and the catabolism of ^{3}H-1,25(OH)$_2$D$_3$ to ^{3}H-1,24,25(OH)$_3$D$_3$ were unexpectedly low ($\leq$5% and $\leq$2%), as compared to the amount of calcitriol generated. Exogenous addition of unlabeled 1,25(OH)$_2$D$_3$, 1,24R(OH)$_2$D$_3$, calcipotriol, or 24R,25(OH)$_2$D$_3$ at concentrations as low as 10^{-11} M, potently inhibited the ^{3}H-1,25(OH)$_2$D$_3$ production. These results suggest that EGF-treated HaCaT keratinocytes could serve for further studies of the vitamin D_3 pathway and its relationship to proliferation and differentiation, but differences in calcitriol synthesis and catabolism from those in cultured primary keratinocytes or other cell lines must be considered. ***Key words: calcidiol/ calcitriol/epidermal growth factor/ keratinocyte. J Invest Dermatol 108:78–82, 1997***

Normal human keratinocytes not only respond to exogenous 1,25-dihydroxyvitamin D_3/calcitriol [$1,25(OH)_2D_3$] with changes in proliferation and differentation (Hosomi *et al*, 1983; Smith *et al*, 1986; McLane *et al*, 1990) but also possess a high capacity to convert exogeneous 25-hydroxyvitamin D_3/calcidiol ($25OHD_3$) to calcitriol and other hydroxylated vitamin D_3 metabolites, e.g., 24R,25-dihydroxyvitamin D_3 [$24R,25(OH)_2D_3$] and 1,24R,25-trihydroxyvitamin D_3 [$1,24R,25(OH)_3D_3$] (Bikle *et al*, 1986a, 1986b; Matsumoto *et al*, 1991). The generation of $1,25(OH)_2D_3$ is regulated by hormones similar to parathormone and calcitriol itself (Bikle *et al*, 1986a; Bikle and Pillai, 1993). Additionally, calcitriol synthesis is regulated by extracellular calcium (van Leeuwen *et al*, 1994), as well as by cytokines such as interferon-γ (Bikle *et al*, 1989) and tumor necrosis factor α (Bikle *et al*, 1991). Moreover, the production of calcitriol changes as the keratinocytes differentiate (Bikle and Pillai, 1993; Pillai *et al*, 1988).

This study was designed to establish a simple and promising cellular model for the investigation of the calcitriol pathway and its modulation using HaCaT cell line. HaCaT (human adult low calcium high temperature) cells are spontaneously transformed human keratinocytes that have the characteristics of basal epidermal keratinocytes (Boukamp *et al*, 1988). This line can be used as an *in vitro* model for highly proliferative epidermis (Ockenfels *et al*, 1995), as well as a model for cells defective in terminal differentiation (Schürer *et al*, 1993). HaCaT cells express nuclear $1,25(OH)_2D_3$ receptors (VDR) (Reichrath *et al*, 1993), indicating that this line should be suitable for analyzing the genomic effects of calcitriol. The link between intracellular calcitriol production and VDR expression might be of importance in the modulation of cell proliferation/differentiation in human skin. Defects in this system could play a role in the pathogenesis of psoriasis, a disease characterized by hyper-proliferation, altered differentation of keratinocytes, and inflammation. Therefore, it was determined (i) whether HaCaT cells could synthesize $1,25(OH)_2D_3$ and (ii) how this synthesis can be modulated by appropriate agents.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from GIBCO (Grand Island, NY). Culture flasks (35- mm) were from Falcon (Heidelberg, Germany). 25-Hydroxy[26,27-methyl-^{3}H]vitamin D_3 (^{3}H-25OHD$_3$, 177 Ci/mmol), 1α,25-dihydroxy[26,27-methyl-^{3}H]vitamin D_3 [^{3}H-1,25(OH)$_2$D$_3$, 173,5 Ci/mmol], and 24R,25-dihydroxy[26,27-methyl-^{3}H]vitamin D_3 [^{3}H-24R,25(OH)$_2$D$_3$, 170 Ci/mmol] were purchased from Amersham (Braunschweig, Germany). 1α,24S(OH)$_2$-22ene-25,27-Cyclopropyl-D$_3$/calcipotriol/MC903 was kindly provided by Dr. Lise Binderup (Leo Pharmaceutical Products, Ballerup, Denmark); 25OHD$_3$, 1,25(OH)$_2$D$_3$, 24R,25(OH)$_2$D$_3$, and 1,24R(OH)$_2$D$_3$, were gifts from Dr. Seiichi Ishizuka (Teijin Limited, Tokyo, Japan; 1,24R,25(OH)$_3$D$_3$ was kindly donated by Hoffmann-La Roche AG (Basel, Switzerland). 1,25(OH)$_2$D$_3$ calf thymus receptor and other reagents for the study of binding properties were from a 1,25(OH)$_2$D$_3$ radioassay kit provided by Nichols Institute (Bad Nauheim, Germany). Solvents for solid-phase extraction (acetonitrile) and high performance liquid chromatography (HPLC) (n-hexane, 2-propanol, and methanol)

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Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; $24R,25(OH)_2D_3$, 24R,25-dihydroxyvitamin D_3; $1,24R,25(OH)_3D_3$, 1,24R,25-trihydroxyvitamin D_3; $25OHD_3$, 25-hydroxyvitamin D_3; IC_{50}, half-maximal inhibitor concentration.

were purchased from Merck (Darmstadt, Germany). Solid-phase, ENCaPharm 100RP 18, 70 μm, was provided by Molnar Institute (Berlin, Germany). The following agents were from Sigma (Deisenhofen, Germany): recombinant human epidermal growth factor (EGF) from *Saccharomyces cerevisiae*, recombinant human transforming growth factor-α (TGF-α) from *Escherichia coli*, actinomycin D, cycloheximide, trypan blue, and gentamycin. Scintillation cocktail, Ready Protein+, was from Beckman Instruments (Fullerton, CA). All other reagents were of analytical grade.

Cell Culture Protocol The human keratinocyte cell line HaCaT (Boukamp *et al*, 1988) was kindly provided by Professor N.E. Fusenig of the German Cancer Research Center (Heidelberg, Germany). Cells were seeded at a density of 5×10^4 cells/cm^2 and grown in DMEM supplemented with 5% (vol/vol) fetal bovine serum and 50 μg gentamycin per ml at 95% relative humidity, 5% CO_2, and 37°C. After 2 d, the medium was replaced for 18 h by serum-free DMEM in order to induce synchronization of the cell cycle. The serum-free DMEM was then replaced by fetal bovine serum–supplemented medium for 6 h, followed by DMEM supplemented with EGF or TGF-α for a further 16 h. At this time, cells were almost confluent (100% confluent monolayers equal to $\sim 1 \times 10^6$ cells). In order to assess whether the basal as well as the induced calcitriol production requires RNA- and/or *de novo* protein synthesis, cells were treated during the incubation (in the absence/presence of EGF) with actinomycin D (final concentration, 2 μg per ml) and cycloheximide (final concentration, 5 μg per ml) prior to the addition of ^{3}H-25OHD$_3$. Investigations into the regulation of the 25OHD$_3$ metabolism were carried out with various concentrations of unlabeled vitamin D$_3$ analogs dissolved in 10 μl of ethanol and added to the cultures immediately after the addition of EGF 16 h prior to the assay. Following the incubation, cells were washed with fresh medium without the vitamin D$_3$ analogs, after which ^{3}H-25OHD$_3$ was added in 10 μl of ethanol to 1.2 ml of medium and further treated as described in the next section. Attached cells from one flask of each assay were counted in a Neubauer hemocytometer, and their viability was determined by trypan blue exclusion. Viability was always ≥ 93%.

Incubation Conditions ^{3}H-25OHD$_3$ (243 pM, 0.05 μCi) was added to the culture flask (1.2 ml of serum-free DMEM) in 10 μl of ethanol. After the incubation period, the supernatant was removed; cells were incubated in 0.6 ml of 0.05% (wt/vol) ethylendiamine tetraacetic acid in phosphate-buffered saline and incubated for 15 min at 37°C. Afterward 1.2 ml of 0.05% (wt/vol) trypsin/0.02% (wt/vol) ethylenediamine tetraacetic acid in phosphate-buffered saline were added for 5 min at 37°C. Detached cells were removed and added to the cell supernatant collected earlier. The culture flask was rinsed twice with 1.5 ml of acetonitrile, which was also added to the cell suspension. This mixture was treated with ultrasound (UD 2, WECO-Optik-Maschinen, Düsseldorf, Germany) for 1 min. After mixing for 1 min, the suspension was centrifuged at $3700 \times g$ for 10 min at 4°C. ^{3}H-Vitamin D$_3$ metabolites in the supernatant were separated by solid-phase extraction using ENCaPharm 100 RP 18. Cartridges were prewashed with 5 ml of methanol followed by 5 ml of aqua dest. After applying the supernatant (6 ml) and washing with 2×1 ml methanol/water (70:30, vol/vol), extracted ^{3}H-vitamin D$_3$ metabolites were eluted with 4 ml of acetonitrile. Eluates were dried under nitrogen, and the residues were dissolved in 250 μl of the HPLC eluent.

Analysis and Identification of Vitamin D$_3$ Metabolites Hydroxylated ^{3}H-vitamin D$_3$ metabolites in 200-μl samples were separated and analyzed by a Merck/Hitachi HPLC system (Merck, Darmstadt, Germany); column: LiChroCART 250–4, Superspher Si60, 5 μm; eluent: n-hexane: 2-propanol:methanol = 87:10:3 (vol/vol/vol); flow rate: 1 ml/min; fractions (1 ml) were collected and mixed with 10 ml of scintillation cocktail, and the radioactivity was determined in a β-counter (LS 6000 LL, Beckman). The results were converted to femtomoles of metabolite per culture flask based on the specific activity of the amount of ^{3}H-25OHD$_3$ originally added to the culture and normalized to 10^6 cells. This calculation assumes that each metabolite generated has the same specific activity as ^{3}H-25OHD$_3$. Therefore, the data represent relative and not absolute counts. The recovery of radioactivity was 86 ± 3% (mean ± SD, n = 5).

The presumptive peaks of 25OHD$_3$, 24R,25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$, and 1,24R,25(OH)$_3$D$_3$ were identified by co-chromatography of ^{3}H-labeled and unlabeled standards. The latter were monitored by ultraviolet detection at 265 nm. For further identification of generated ^{3}H-1,25(OH)$_2$D$_3$, fractions containing ^{3}H-labeled calcitriol were pooled and evaporated, and the residue was rechromatographed on HPLC using a Hibar column, 250–4, LiChrospher 100RP-18, 5 μm, (Merck, Darmstadt, Germany); eluent: methanol:water = 85:15 (vol/vol); flow rate: 1 ml/min. Fractions (1 ml) were collected and mixed with 10 ml of scintillation cocktail, and radioactivity was determined as described above. Additionally, the identity of generated ^{3}H-1,25 (OH)$_2$D$_3$ was shown by its affinity to the calf thymus receptor (Hollis, 1986). Briefly, the presumptive peaks of ^{3}H-1,25(OH)$_2$D$_3$ from four separate experiments were collected and dried, and the residue was dissolved in 250 μl of phosphate-buffered 2-propanol [zero standard of the 1,25 (OH)$_2$D$_3$ radioassay kit] and used as a tracer. Standards of calcitriol (0, 50, 100, and 200 pg per ml, 50 μl each) were pipetted in four tubes. After addition of 500 μl of a buffered solution of calf thymus receptor, tubes were incubated for 1 h at room temperature. Subsequently, 50 μl of the prepared tracer (total counts: ≈3000 cpm; maximum binding: ≈1100 cpm) were transferred into the tubes and further incubated for 1 h. The bound ligand was separated from the unbound ligand with dextran-coated charcoal within 30 min at 4°C, and the suspension was centrifuged at $1500 \times g$ for 20 min at 4°C. Supernatants were transferred to scintillation vials, and, after addition of 10 ml of scintillation coctail, the radioactivity of the tritiated receptor–bound calcitriol was measured in a β-counter as described above. For further characterization of putative ^{3}H-24,25(OH)$_2$D$_3$ and ^{3}H-1,24,25(OH)$_3$D$_3$, fractions from four separate experiments were pooled, divided in two equal parts, and dried. The residues were dissolved in 100 μl of methanol and treated with 100 μl of a 5% aqueous solution of sodium metaperiodate ($NaIO_4$) for 30 min at 25°C as described by Tanaka *et al* (1981). After that, the samples were dried again under a stream of nitrogen, and the residues were redissolved in 100 μl of methanol. Next, 10 ml of scintillation cocktail were added, and the radioactivity was determined.

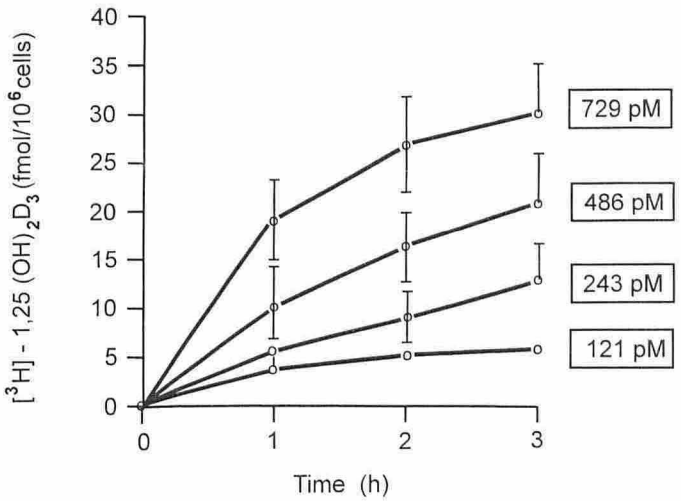

Figure 1. Time course of the synthesis of ^{3}H-1,25(OH)$_2$D$_3$ in HaCaT cells. Confluent cultures were incubated with different concentrations of ^{3}H-25OHD$_3$ (121–729 pM) for 1 h. Cells and medium were extracted together and analyzed for the amount of ^{3}H-1,25(OH)$_2$D$_3$ by HPLC. Values are given as mean ± SD of three independent experiments.

Statistical Analysis Results are presented as means ± SD. Data were analyzed by one-way analysis of variance to assess statistical significance between means. Differences between the means were considered statistically significant at $p < 0.05$ using the Bonferroni method and the GraphPad Instat computer program (version 2.0) (Intuitive Software for Science, San Diego, CA).

RESULTS

Metabolism of ^{3}H-25OHD^3 As shown in **Fig 1**, the addition of different concentrations of ^{3}H-25OHD$_3$ (121 pM, 243 pM, 486 pM, and 729 pM) to confluent HaCaT keratinocytes cultured without addition of growth factors in the terminal phase of cultivation resulted in a dose-dependent production of ^{3}H-1,25(OH)$_2$D$_3$, but the levels of calcitriol generated were unexpectedly low. The addition of EGF or TGF-α to the almost confluent cells increased the production of calcitriol. At a concentration of 243 pM ^{3}H-25OHD$_3$, the synthesis of calcitriol after 1 h was increased almost 10-fold after addition of 1 nM EGF or 10 nM TGF-α as compared to controls **(Fig 2)**. The stimulation response to these growth factors shows different kinetics. The course of 1-hydroxylation of calcidiol after treatment with different concentrations of EGF is best described by a sigmoidal plot, whereas the

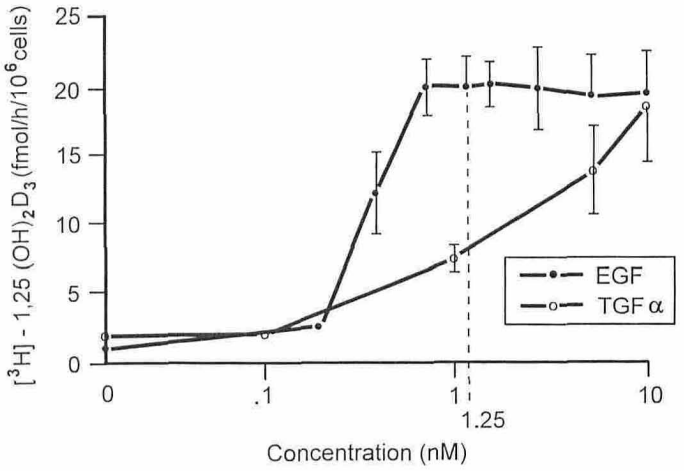

Figure 2. ^{3}H-1,25(OH)$_2$D$_3$ production by cultured HaCaT cells in dependence on growth factor concentration. Cells were grown at different concentrations of EGF and TGF-α (0–10 nM) and incubated with 243 pM ^{3}H-25OHD$_3$ for 1 h. Values are mean ± SD of three (EGF) and two (TGF-α) experiments, respectively.

effect of TGF-α rather resembles an exponential curve. EGF (1.25 nM) was used in further experiments with 243 pM ^{3}H-25OHD$_3$. The time course of stimulation by EGF showed an almost linear increase of calcitriol production up to 4 h **(Fig 3)**. In all following experiments, incubation time was set to 3 h. The synthesized ^{3}H-1,25(OH)$_2$D$_3$ remained predominantly (≥75%) in the cells, as determined by separate processing of cells and cell supernatant (data not shown).

Figure 4 illustrates that the calcitriol synthesis in preconfluent cells stimulated by EGF is significantly ($p < 0.01$) higher compared to confluent cultures (≈1 × 10^6 cells/flask, ≈100% confluent). A similar effect was observed for cells grown without EGF **(Fig 4)**. Despite the lower calcitriol synthesis in confluent compared to preconfluent cells, the former were used because of the higher reliability of cell number determination at higher cell densities.

A representative radiochromatogram is shown in **Fig 5**. Characteristic elution times were 7 min for ^{3}H-25OHD$_3$, 11 min for ^{3}H-24R,25(OH)$_2$D$_3$, and 20 min for ^{3}H-1,25(OH)$_2$D$_3$. The elution time for 1,24R,25(OH)$_3$D$_3$ (^{3}H-1,24R,25(OH)$_3$D$_3$ was not available as a standard) was close to 40 min. The identity of the substance(s) in fraction 5 (FR 5), which are impurities of ^{3}H-

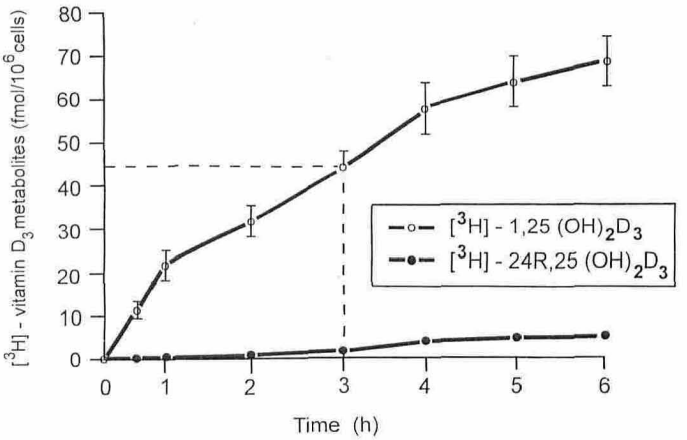

Figure 3. The generation of ^{3}H-1,25(OH)$_2$D$_3$ and ^{3}H-24R,25(OH)$_2$D$_3$ as a function of time. HaCaT cells grown at 1.25 nM EGF were incubated with 243 pM ^{3}H-25OHD$_3$ for the indicated times. Data represent mean ± SD from three separate experiments.

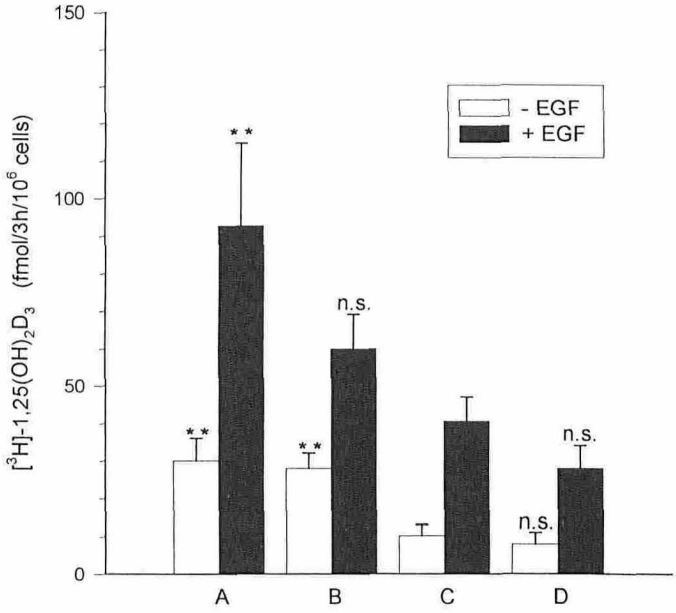

Figure 4. Dependence of the calcitriol synthesis on the degree of confluence of keratinocyte line. HaCaT cells were grown with 1.25 nM EGF or without EGF and incubated with 243 pM ^{3}H-25OHD$_3$ for 3 h. The degree of confluence (A–D) corresponds to cell numbers of: A (≈50% confluent): ≈0.3 × 10^6; B (≈75% confluent): ≈0.6 × 10^6; C (≈100% confluent): ≈1 × 10^6; and D (postconfluent with stratifications): ≈2.2 × 10^6. Data are expressed as mean ± SD of three independent experiments and analyzed by analysis of variance (**, $p < 0.01$ to column C (±EGF); ns, not significant).

25OHD$_3$ used, and fraction 17 (FR 17) are unknown. After 3 h, the production of ^{3}H-24R,25(OH)$_2$D$_3$ increased to approximately 5% of the calcitriol synthesized. The generation of ^{3}H-1,24R,25(OH)$_3$D$_3$ was lower than 1 fmol (≤2% of generated calcitriol) (data not shown in detail). The further conversion of ^{3}H-1,24R,25(OH)$_3$D$_3$ finally to ^{3}H-calcitroic acid was not investigated. Rechromatography of the presumptive peak with ^{3}H-labeled calcitriol indicated identity with the corresponding ^{3}H-labeled standard [elution time for ^{3}H-1,25(OH)$_2$D$_3$ under these conditions was 7 min, radiochromatogram not shown]. Further

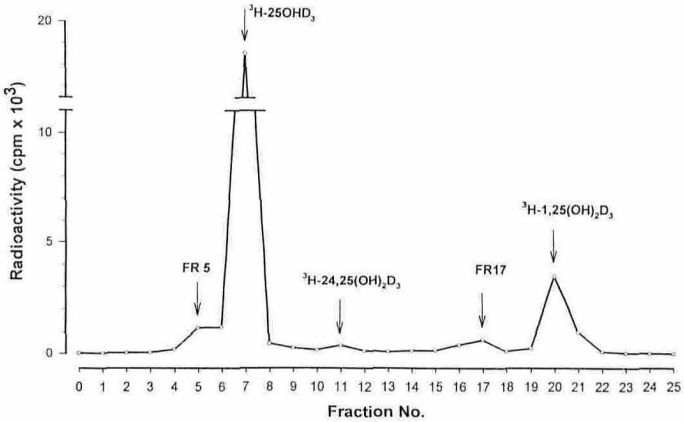

Figure 5. Typical radiochromatogram of the solid-phase extract of cultured HaCaT cells (cells plus medium). Cells were grown at 1.25 nM EGF and incubated with 243 pM ^{3}H-25OHD$_3$ for 3 h. Straight-phase HPLC using a Superspher Si 60 column was performed with a mobile phase of 10% 2-propanol and 3% methanol in n-hexane; fraction volume, 1.0 ml. *Arrows* depict elution positions of ^{3}H-labeled D$_3$ standards in control separations.

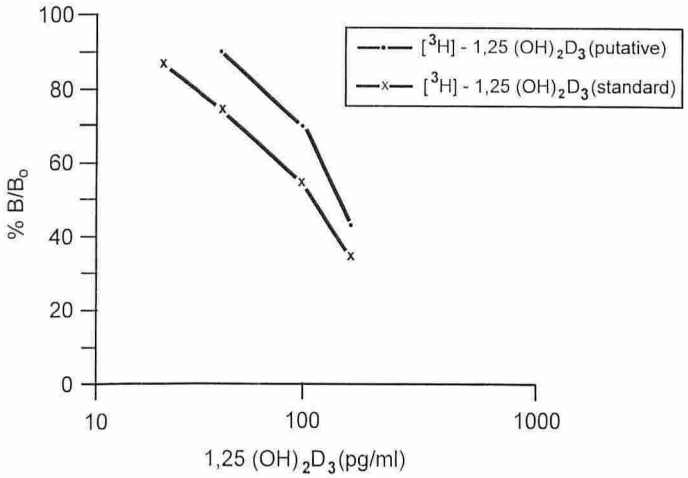

Figure 6. Comparison of the binding of putative and authentic ^{3}H-1,25(OH)$_2$D$_3$ to calf thymus receptor. The receptor was first incubated with different concentrations of unlabeled authentic calcitriol (control, 50, 100, and 200 pg per ml) for 1 h followed by further incubation with putative and authentic ^{3}H-1,25(OH)$_2$D$_3$, respectively, for 1 h. Presumptive and authentic ^{3}H-1,25(OH)$_2$D$_3$ have comparable affinity for the calf thymus receptor. The means of duplicate determinations are shown.

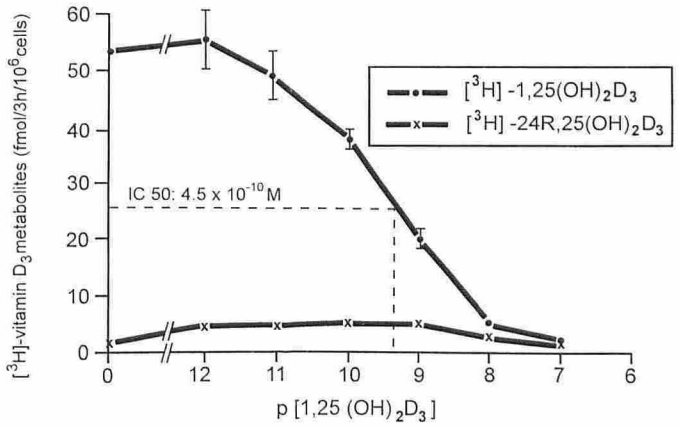

Figure 7. Effect of 1,25(OH)$_2$D$_3$ on hydroxylation of ^{3}H-25OHD$_3$. HaCaT cells grown at 1.25 nM EGF were incubated with the indicated concentrations of unlabeled 1,25(OH)$_2$D$_3$ 16 h prior to addition of ^{3}H-25OHD$_3$. The medium was removed, and the cells were washed once with serum-free medium and subsequently incubated with 243 pM ^{3}H-25OHD$_3$ for 3 h. The production of ^{3}H-1,25(OH)$_2$D$_3$ and ^{3}H-24R,25(OH)$_2$D$_3$ is shown; p[1,25(OH)$_2$D$_3$] is the negative log of 1,25(OH)$_2$D$_3$ molarity. Data are mean ± SD of three independent experiments.

identification of presumptive ^{3}H-1,25(OH)$_2$D$_3$ was carried out by demonstrating its specific binding to the calf thymus receptor. As depicted in **Fig 6**, both ^{3}H-tracers were bound and the radioactivity measured was inversely related to the amount of unlabeled calcitriol standard. Putative ^{3}H-24,25(OH)$_2$D$_3$ and ^{3}H-1,24,25(OH)$_3$D$_3$ were further characterized by their reaction with sodium periodate as shown in **Table I**. Loss of radioactivity indicates specific cleavage of these vicinal diols, which are labeled at C-26,27.

In order to determine whether production of calcitriol requires transcriptional activity and/or protein biosynthesis, the confluent density-arrested HaCaT cells were grown with or without EGF and incubated separately with 2 μg actinomycin D per ml and 5 μg cycloheximide per ml. Basal synthesis of ^{3}H-1,25(OH)$_2$D$_3$ was only slightly inhibited by actinomycin D. In contrast, cycloheximide inhibited calcitriol synthesis by 36 ± 3% (n = 2) when compared to controls. The generation of calcitriol in EGF-stimulated HaCaT cells was reduced by 76 ± 2% (n = 3) when treated with actinomycin D, whereas cycloheximide reduced ^{3}H-1,25(OH)$_2$D$_3$ recovery by 70.7 ± 2.1% (n = 3). The viability of the cells was not changed by these agents within 24 h.

Effects of Vitamin D$_3$ Derivatives on Hydroxylation of ^{3}H-25OHD$_3$ To determine whether EGF-stimulated hydroxylation of ^{3}H-25OHD$_3$ is regulated by vitamin D$_3$ metabolites and analogs, the effects of some selected unlabeled vitamin D$_3$ derivatives were examined. When unlabeled 1,25(OH)$_2$D$_3$ was added to the cell culture 16 h prior to the addition of the substrate ^{3}H-25OHD$_3$, the recovery of ^{3}H-1,25(OH)$_2$D$_3$ was reduced in a concentration-dependent manner **(Fig 7)**. The half-maximal inhibitor concentration (IC_{50}) was approximately 4.5×10^{-10}M. Only small amounts of ^{3}H-24R,25(OH)$_2$D$_3$ (≤10% of generated calcitriol) and of ^{3}H-1,24R,25(OH)$_3$D$_3$ (≤4% of generated calcitriol) were synthesized when the cells were preincubated with increasing doses of 1,25(OH)$_2$D$_3$. The effects of calcipotriol, tacalcitol (1,24R(OH)$_2$D$_3$), and 24R,25(OH)$_2$D$_3$ were tested in the same manner. As shown in **Table II**, 1,24R(OH)$_2$D$_3$ inhibited the synthesis of ^{3}H-1,25(OH)$_2$D$_3$ most potently and calcipotriol least potently. The effect of these vitamin D$_3$ analogs on the synthesis of ^{3}H-24R,25(OH)$_2$D$_3$ and ^{3}H-1,24R,25(OH)$_3$D$_3$ was similar to that of calcitriol (data not shown in detail).

Table I. Cleavage of putative ^{3}H-24,25(OH)$_2$D$_3$ and ^{3}H-1,24,25(OH)$_3$D$_3$ by Periodate[a]

Metabolite	Control (cpm)	Radioactivity after Treatment with $NaIO_4$ (cpm)	Loss of Radioactivity (%)
^{3}H-24,25(OH)$_2$D$_3$	1180	60	95
^{3}H-1,24,25(OH)$_3$D$_3$	368	29	92

[a] Effect of periodate on vicinal diols at C-24 and C-25. Fraction 11 (putative ^{3}H-24,25(OH)$_2$D$_3$) and fractions 40/41 (putative ^{3}H-1,24,25(OH)$_3$D$_3$) from *four* separate experiments as described in **Fig 5** were pooled, and divided in two equal parts. The samples were prepared for treatment with an aqueous solution of $NaIO_4$ or water (controls). Radioactivities were determined as described above.

DISCUSSION

Our results demonstrate that HaCaT cells are capable of producing 1,25(OH)$_2$D$_3$, but only in small amounts. In accordance with our results, Smith and Holick (1985) as well as Matsumoto *et al* (1991) reported only minor calcitriol synthesis by cultured keratinocytes from adult donors. Bikle *et al* (1986a), however, demonstrated that human foreskin keratinocytes grown on feeder 3T3 cells metabolize substantial amounts of ^{3}H-25OHD$_3$ to ^{3}H-1,25(OH)$_2$D$_3$ and other metabolites.

It is difficult to understand why TGF-α and EGF, which are

Table II. Effect of Various Vitamin D$_3$ Derivatives on the 1-Hydroxylation of ^{3}H-25OHD$_3$[a]

Vitamin D$_3$ Derivative	IC_{50} (pM)
1,24R(OH)$_2$D$_3$	45 ± 9
1,25(OH)$_2$D$_3$	450 ± 56
24R,25(OH)$_2$D$_3$	600 ± 63
Calcipotriol	630 ± 79

[a] Comparison of four vitamin D$_3$ derivatives with regard to their ability to inhibit ^{3}H-1,25(OH)$_2$D$_3$ production. HaCaT cells were incubated together with 1.25 nM EGF and nonradioactively labeled vitamin D$_3$ derivatives at different concentrations (control and 10^{-12} to 10^{-7} M) for 16 h. The medium was removed, and the cells were washed once with fresh medium and then incubated with ^{3}H-25OHD$_3$ for 3 h. Recoveries of ^{3}H-1,25(OH)$_2$D$_3$ (fmol/3 h/10^6 cells) were plotted against the negative log of vitamin D$_3$ derivative concentration. The data for IC_{50} (half-maximal inhibitor concentration) are expressed as mean ± SD of three separate experiments for each vitamin D$_3$ derivative.

similar in structure and share the same receptor, have such different effects on calcitriol synthesis. Notably, EGF and TGF-α differ markedly in their ability to be internalized by the EGF receptor (Ebner and Derynck, 1991), which would offer a plausible explanation for the different effects of EGF and TGF-α on calcitriol synthesis.

Possibly as a result of a lack of substrate and/or self-inhibition of 1-hydroxylase by generated ^{3}H-1,25(OH)$_2$D$_3$, the rate of calcitriol synthesis declines after 4 h. In primary neonatal keratinocytes, a maximum conversion rate has been found after 1 h (Bikle *et al*, 1986a). Using culture-grown neonatal keratinocytes, Ray *et al* (1995) have demonstrated a rapid metabolic degradation of ^{3}H-1,25(OH)$_2$D$_3$ via ^{3}H-1,24R,25(OH)$_3$D$_3$ to ^{3}H-calcitroic acid within a few hours. In our experiments, the relative constant concentration of ^{3}H-1,25(OH)$_2$D$_3$ after 6 h of incubation shows that catabolism of generated ^{3}H-1,25(OH)$_2$D$_3$ via this pathway appears to be negligible. The amount of ^{3}H-1,25(OH)$_2$D$_3$ generated after 3 h ($\approx$45 pM) is only sufficient to inhibit its own production to approximately 80% (IC_{50} = 450 pM, see **Table II** and **Fig 7**). In accordance with Bikle *et al* (1986a), we found that comparatively little ^{3}H-1,25(OH)$_2$D$_3$ was released from cells into the medium.

Experiments using different degrees of cell confluence showed that the production of calcitriol in poorly differentiated HaCaT cells, similar to primary neonatal keratinocytes (Bikle and Pillai, 1993), is higher than in terminally differentiated cells. This effect is markedly increased when cells are pre-treated with EGF. These observations support the finding of the modulation of proliferation and differentiation processes in keratinocytes by intracellular calcitriol synthesis, and vice versa (Bikle and Pillai, 1993).

It has been demonstrated with cultured keratinocytes that growth stimulation by EGF or TGF-α is an indirect effect initiated by an increase in cellular migration from the edge of large colonies (Barrandon and Green, 1987). On the other hand, EGF can induce the *de novo* synthesis of proteins including enzymes (Bruns *et al*, 1989; Olson *et al*, 1990). Experiments using actinomycin D indicated that EGF-induced calcitriol synthesis requires transcriptional activity. Furthermore, we demonstrated that cycloheximide clearly blocked the basal and EGF-induced production of ^{3}H-1,25(OH)$_2$D$_3$ in our system, indicating that the generation of calcitriol requires protein biosynthesis. Because of the poor correlation between the growth-stimulatory effect of EGF at $\approx$100% confluence (equivalent to nearly constant cell numbers) and calcitriol synthesis, it is assumed that EGF causes a *de novo* synthesis of intracellular 1-hydroxylase. In addition, other reports have shown that the inhibition of protein biosynthesis by cycloheximide decreases EGF receptor–binding activity (Carpenter and Cohen, 1979). It is concluded that the half-lives of the 1-hydroxylase and/or of the EGF receptor seem to be relatively short. Aharonov *et al* (1978) estimated the half-life of the EGF receptor in 3T3 cells to be 6 h in the presence of cycloheximide.

When unlabeled vitamin D$_3$ derivatives were added to the cell culture 16 h prior to the addition of the ^{3}H-labeled substrate, the recovery of tritiated calcitriol was markedly reduced. The mean IC_{50} of calcitriol amounts to 4.5×10^{-10}M, which is close to the value obtained with primary neonatal keratinocytes (10^{-11}M) (Bikle *et al*, 1986a). Low levels of ^{3}H-24R,25(OH)$_2$D$_3$ and ^{3}H-1,24R,25(OH)$_3$D$_3$ (data not shown) were unexpected, because Bikle *et al* (1986a) had found that calcitriol increases its own catabolism. 1,24R(OH)$_2$D$_3$ (mean IC_{50} = 45 pM) was the most potent, and calcipotriol (mean IC_{50} = 630pM) was the least potent inhibitor. The relative high potency of 24R,25(OH)$_2$D$_3$ (IC_{50} = 600 pM) was surprising, because among natural vitamin D$_3$ metabolites only calcitriol is known to exhibit a negative feedback effect on 1-hydroxylase (Bikle *et al*, 1986a).

In summary, our study demonstrates that HaCaT cells are capable of hydroxylating 25OHD$_3$ to 1,25(OH)$_2$D$_3$ and, to a small extent, to other vitamin D$_3$ metabolites. We demonstrate that hydroxylation is augmented by EGF and TGF-α. Therefore, the HaCaT line appears to be a suitable model for the investigation of calcitriol pathways and their implications on the growth and differentiation of keratinocytes. Although HaCaT cells offer certain advantages to dermatologic research, the differences in vitamin D$_3$ metabolism to cultured primary or transformed/tumorigenic keratinocytes (Bikle *et al*, 1991) must be considered when using this cell line.

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